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## Novel DNA structures resulting from dTam3 excision in tobacco

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**Key words:** transposition, Tam3, two-element system, excision sites, rearrangements

### Abstract

A Tam3 two-element system has been designed by combining an immobilized Tam3 element with a non-autonomous dTam3 element inserted into the HPT gene. The phenotypic assay employed, restored hygromycin resistance, indicated that *trans*-activation of the non-autonomous dTam3 element occurred. Molecular analyses of the excision sites revealed that the ends of the dTam3 element remain in the empty donor sites. The predominant consequence of this type of excision appears to be that excised fragments fail to re-integrate into the tobacco genome. Only one case of dTam3 re-integration could be detected. The ends of this element had been degraded upon integration into the tobacco genome. Either the altered structure of the Tam3 derivatives or tobacco host factors are influencing the *trans*-activation of a dTam3 element, resulting in aberrant excision.

### Introduction

The plant transposable elements Ac, Spm (from maize) and Tam1 and Tam3 (from snapdragon) are thought to transpose by a mechanism which involves excision of a single copy of the transposon from one site in the plant genome and re-integration of the same copy at a new site. During this process all of these transposable elements induce a target site duplication upon integration into the host genome [3, 6, 20, 25]. Perfect excision of a plant transposable element would thus generate a direct repeat of this sequence, but frequently, if not always, an altered sequence is generated as a result of imprecise repair of the empty

donor site by the DNA repair mechanisms of a plant cell. These empty donor site changes include deletions, inversions or additions of several base pairs [2, 6, 31]. One model explaining these small-scale rearrangements proposes that upon excision the DNA repair mechanism induces these changes, while repairing the staggered cut made by the transposase. This model predicts that an extra-chromosomal form of the transposon would occur after cleavage at both termini during excision and prior to re-integration. This extra-chromosomal form, however, could be very transitory. In the case of the maize transposable element Mu extra-chromosomal circles have been detected which might represent such intermedi-

ates and, more recently, PCR analysis of dSpm transposition in tobacco has provided evidence for a linear dSpm intermediate of transposition [23].

An alternative model has been proposed by Robbins *et al.* [26], based on large-scale rearrangements induced by Tam3 transposition. This model proposes that the donor and recipient site of the host chromosome become associated during the transposition process. In the course of excision cleavage occurs at both termini sequentially. The first end is cut and becomes ligated to the new site of insertion prior to cleavage at the second end. In this case no extra-chromosomal form of the transposon occurs. Not only does this model explain why transposable elements can cause large-scale rearrangements and chromosome breakage, it also provides an explanation for the preferential transposition of the plant transposable elements Ac, Spm and Tam3 to closely linked sites on the chromosome [9, 16, 17]. As Ac retains this property in a new host (tobacco) it can be envisaged that this behaviour is inherent in the transposition mechanism.

With the introduction of Ac into new hosts it became clear in subsequent analyses that the maize Ac and Ds elements retain most of their properties in heterologous hosts [1, 11, 13, 14]. On the contrary, a Tam3 two-element system appears to be unable to perform the transposition process with the same efficiency and accuracy as the autonomous Tam3 element in both tobacco and *Antirrhinum* [20, 21]. A non-autonomous Tam3 element (dTam3), which is activated *in trans* by a 'wings-clipped' Tam3 element (Tam3  $\Delta$ TIR), produces rearrangements in the HPT gene it excises from, while re-integration of the dTam3 element was detected in only one out of six plants analysed [12]. In this paper we present the analysis of the empty donor sites produced by aberrant dTam3 excision. Only one re-integrated dTam3 element could be recovered from the tobacco genome. Analysis of this dTam3 element and its flanking sequences indicates deletion of the ends of the transposable element and integration by a process different from transposon-induced integration.

## Materials and methods

### *Plant materials and transformation*

Transgenic tobacco plants were used for a second round of *Agrobacterium* transformation. Plant numbers 2001, 2002 and 2008 harbour a single copy of the T-DNA from plasmid pTT21806 (Tam3  $\Delta$ TIR). Plant number 2105 harbours the T-DNA of plasmid pTT21840 (dTam3). Plants were selected from a F1 progeny by DNA gel blot analysis and grown in the greenhouse prior to transformation. Leaf discs of the tobacco plants were incubated with *Agrobacterium* LBA4404 strains containing plasmid pTT21808 (Tam3  $\Delta$ TIR), in the case of plant 2105 and plasmid pTT21840 (dTam3-HPT) in the case of plants 2001, 2002 and 2008. Selection was carried out on medium containing hygromycin (20–40 mg/l) [10, 12].

### *Molecular techniques*

DNA gel blots were performed as described [10]. The polymerase chain reaction [28] was carried out to amplify the empty donor site fragments from hygromycin-resistant plants using a CaMV 35S primer (GGCTGCAGTCTCCACTGACG-TAAGGGATGACG), a reverse HPT primer (AGCTGAAAGCA CCGAGAATTCTTCGC) and the following thermal cycle: denaturation 94°C 30 s, annealing 55°C 30 s, extension 72°C 1 min, performed 30 times. Fragments were gel-purified, amplified for a second time using the same cycle and cloned into M13 mp18 taking advantage of the *Pst* I and *Eco* RI sites of the primers. Dideoxy sequencing [29] was performed with Klenow polymerase and <sup>32</sup>S dATP or with Taq-polymerase and fluorescently labelled M13 primers employing a DNA sequencer (Applied Biosystems).

### *Electroporation of Escherichia coli MC1061*

DNA of plant 4202 was digested with *Hind* III or with *Eco* RV and 20 µg was ligated in 500 µl re-

action volume with 20 units T4-ligase, 16 h at 16 °C in order to circularize the plant DNA fragments. After precipitation and dialysis the DNA was resuspended in 20  $\mu$ l distilled water and mixed with electro-competent *E. coli* MC1066 cells in 40  $\mu$ l. Using the Biorad Gene Pulser set to 20 kV/cm and 0.1 cm cuvettes (Biorad), the plant DNA mixture was electroporated into the *E. coli* cells [4]. Transformants were selected on chloramphenicol-containing medium (20 mg/ml).

## Results

Upon combination of a non-autonomous element and an immobilized Tam3 element, both inserted into a HPT gene, hygromycin-resistant tobacco plants can be obtained (Fig. 1). As neither of these elements can excise autonomously [10] these results indicate that the non-autonomous dTam3 element is activated *in trans* by the immobilized element Tam3  $\Delta$ IR. To confirm the excision of the dTam3 element from the HPT gene a DNA

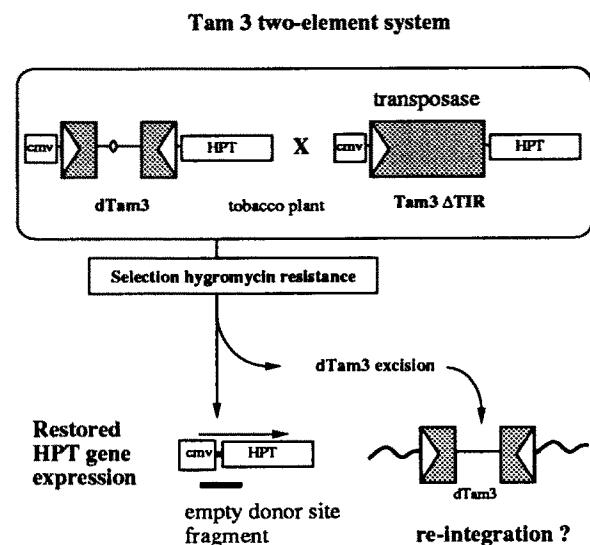


Fig. 1. A Tam3 two-element system: *trans*-activation of a non-autonomous dTam3 element from the HPT gene. Combination of the components of the Tam3 two-element system is achieved by successive transformation of transgenic tobacco plants. Direct selection on hygromycin-containing medium results in recovery of plants where excision of the non-autonomous element has occurred.

gel blot of hygromycin-resistant plants was performed. The *Eco* RI/*Eco* RV-digested DNA of these plants was hybridized with a 0.4 kb CaMV 35S-HPT gene probe, spanning the empty donor site (Fig. 2). As expected none of the fragments that hybridized with this probe had the original size of 0.4 kb, because upon perfect excision of the dTam3 element 64 base pairs flanking the element remain in the empty donor site. However, most fragments representing an empty donor site differ from the predicted 0.46 kb. Plant 3902 contains a smaller fragment, plants 4004, 4202 and 4203 contain larger fragments. Only plants 3903 and 4002 contain fragments of the expected size. The results from this DNA gel blot were confirmed by a PCR experiment with a CaMV 35S primer flanking the *Eco* RV site and a reverse HPT primer containing the *Eco* RI site. No PCR

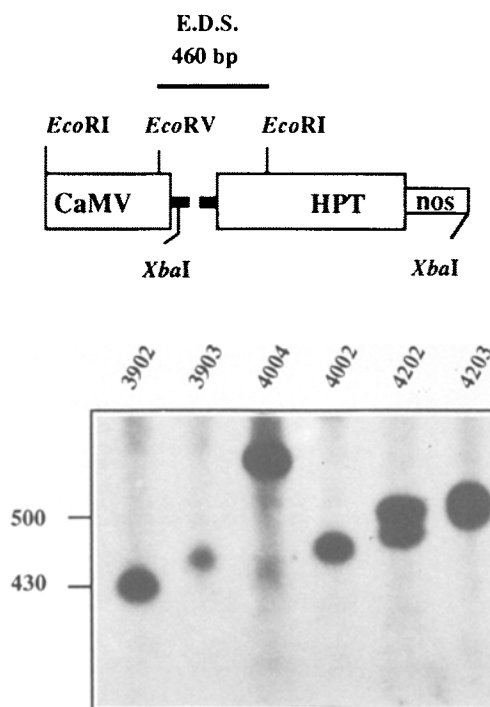


Fig. 2. DNA gel blot analysis of empty donor site fragments of hygromycin-resistant plants. A. Restriction endonuclease map of the chimaeric CaMV 35S-HPT-nos gene. Upon excision of the dTam3 element a 460 bp *Eco* RI-*Eco* RV fragment is expected (E.D.S., empty donor site). B. DNA of independent hygromycin-resistant plants was digested with *Eco* RI/*Eco* RV and the DNA gel blot probed with the E.D.S. fragment indicated in A. Fragment sizes are in base pairs.

products could be detected in DNA from *Agrobacterium* strains harbouring either plasmid pTT21808 or pTT21840 (results not shown). The PCR fragments were cloned into M13mp18 and sequenced (Fig. 3). A perfect excision of the dTam3 element would generate a sequence with a target site duplication of the flanking DNA. Surprisingly, three of the five fragments sequenced contained the terminal inverted repeats and part of the ends of dTam3. The dTam3 element of plants 4202, 4203 and 3903 has excised leaving 25–30 bp from the right end of the element and at a variable part of the left end of the dTam3 element (the left end sequence of the empty donor site of plant 3903 does not originate from Tam3 nor from flanking sequences). The empty donor site of plant 3902 contains no Tam3 sequences nor part of the *pallida* flanking sequences that are expected to remain upon excision of dTam3.

These sequence data reveal that none of the sequences produced upon dTam3 excision matches the empty donor site for autonomous Tam3 excision in *Antirrhinum* or tobacco [20, 21].

Closer inspection of the left part of the empty donor site from plant 4002 reveals that this fragment has resulted from the excision of the 'wings-clipped' Tam3ΔTIR. This can be deduced from the sequence of the left end of the excision site because the Tam3 end fits the deletion that had been introduced by Bal31 deletion [10]. This is a surprising observation as we were unable to detect excision (hygromycin resistance) in control experiments with both pTT21808, pTT21806 (Tam3ΔTIR) and pTT21840 (dTam3). These control experiments were performed in duplicate with the same number of leaf discs from untransformed tobacco plants as in the *trans*-activation experiments, employing the same selection pro-

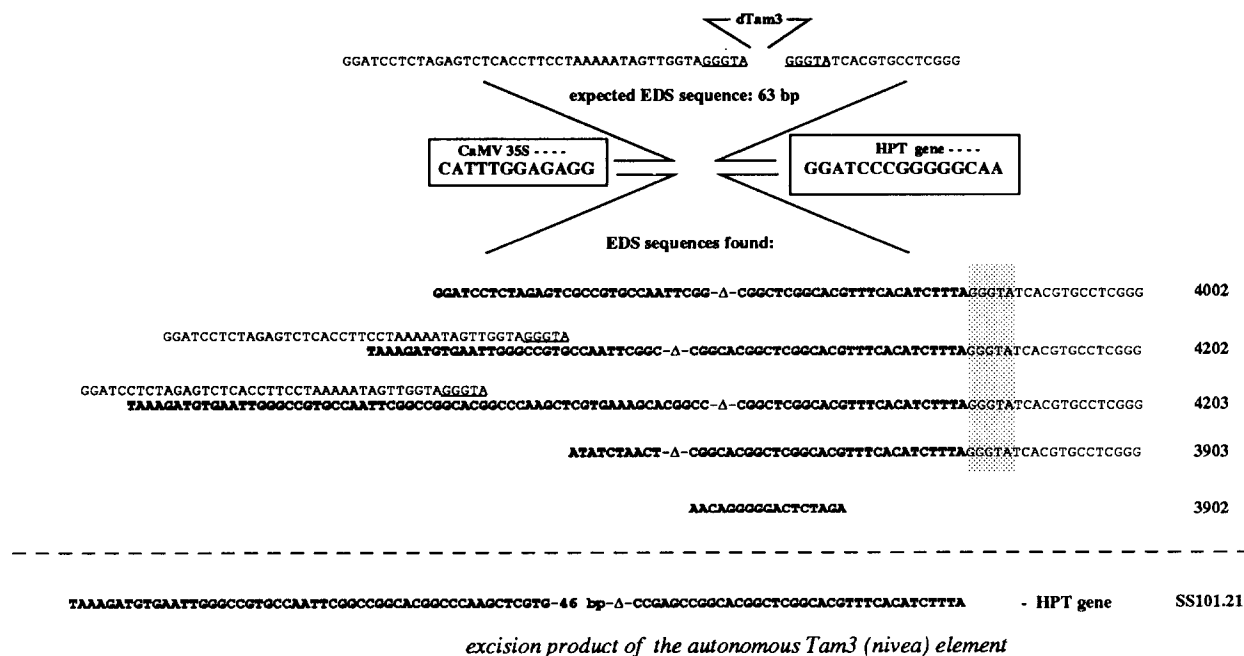


Fig. 3. DNA sequence of dTam3 excision sites. The chimaeric CaMV-HPT gene is projected as two boxes with the last bases of the promoter region and the first bases of the structural gene. The 64 bp DNA flanking the dTam3 element prior to excision are depicted in the top half of the figure, with the target site duplication underlined. The five dTam3 excision sites sequenced have been aligned by the right target site duplication (shaded box). The breakpoints have been indicated by -Δ-. The DNA sequence that is different from the expected empty donor site is printed in bold face. The bottom half of the figure illustrates the excision product of an autonomous Tam3 element from a chimaeric nos-HPT gene (for details, see Discussion). This sequence is aligned to the other dTam3 excision sites by its right-end inverted repeat.

cedure. The case of Tam3 $\Delta$ TIR excision described above is apparently a rare event.

#### *Re-integration of the dTam3 element*

As the excision of the dTam3 elements from the HPT gene appears to occur through a process different from the mechanism of autonomous Tam3 transposition in *Antirrhinum* or tobacco [21], it is interesting to see whether the dTam3 elements re-integrated into the genome of the new host. From DNA gel blot analyses it could be deduced that only in plant 4202 the dTam3 element had re-integrated (data not shown and [12]). To establish whether this integration had taken place in a genetically linked site, hygromycin-resistant progeny of a selfing were screened for cross-over events by DNA gel blot analysis. A cross-over event should be visible as the loss of the re-integrated dTam3 fragment in the Tam3 hybridization pattern of these plants. The analysis indicated that two other T-DNA copies carrying non-transposed dTam3 elements were not linked to the hygromycin resistance trait, as these bands were absent in the DNA of some plants (Fig. 4, lanes 4, 5 and 7). Because plant 4202 is homozygous for the activator Tam3 $\Delta$ TIR element all progeny contain the fragments characteristic of this element. In a total of 48 progeny plants no loss of the re-integrated dTam3 element could be detected. Therefore it can be concluded that the hygromycin resistance trait (the excision site) and the re-integrated dTam3 element are closely linked.

#### *Recovery of the transposed dTam3 element of plant 4202 by electroporation*

As the dTam3 element employed is equipped with plasmid pACYC184, it should be possible to recover the integrated dTam3 element from the plant genome. This can be accomplished by cutting the plant DNA with a restriction enzyme that does not cut inside the dTam3 element, circularization of the fragments and subsequent electroporation of *E. coli* with this mixture [4]. Only

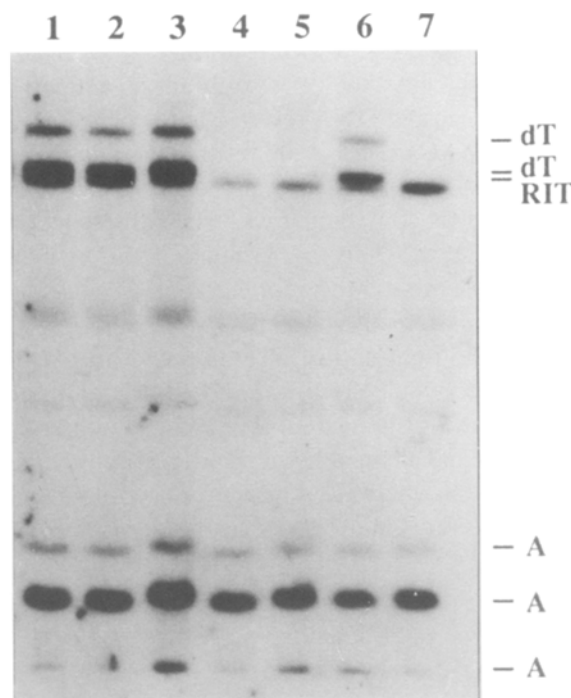


Fig. 4. Assessment of the genetic distance of a newly integrated dTam3 element: DNA gel blot analysis of segregating progeny of plant 4202. DNA (10  $\mu$ g) of individual hygromycin-resistant plants from a selfing of plant 4202 was digested with *Eco* RV and the DNA gel blot was hybridized with a Tam3 probe. Plant 4202 contains an *activator* Tam3 element (bands A) two non-transposed dTam3 elements (bands dT) and a newly integrated dTam3 element (band RIT). The two non-transposed elements are not linked to the hygromycin resistance trait as can be deduced from the absence of these bands in the DNA of some plants (lanes 4, 5 and 7). The re-integrated dTam3 element is linked to the hygromycin resistance trait as it is present in all plants (band RIT).

plasmid sequences will be maintained in *E. coli* and they can be selected for on chloramphenicol-containing medium (the selection marker of pACYC184). To avoid the recovery of the two non-transposed dTam3 copies from plant 4202, a plant harbouring only the transposed dTam3 element and the activator element was selected from the progeny (Fig. 4, lane 4). Restriction enzyme digestion with *Hind* III and *Eco* RV yielded a re-integrated dTam3 fragment of 10 and 7 kb respectively. Other enzymes yielded fragments larger than 15 kb, which are unsuitable for efficient electroporation of *E. coli*. DNA of the se-

lected plant was digested with *Hind* III or *Eco* RV, ligated and electroporated into *E. coli* MC1066. Only the electroporation with the *Eco* RV-digested DNA resulted in the recovery of plasmid pACYC184 with dTam3 and flanking sequences. A physical map of this plasmid, pRIT65, was constructed with several restriction enzymes (Fig. 5). From these data it was evident that both ends of the re-integrated dTam3 element had been altered. The *Pvu* II and *Hae* II sites of the right end of dTam3 could no longer be detected, while the *Sma* I and *Bgl* I sites of the left end had disappeared. Apparently both ends of the dTam3 element had been degraded beyond the position where it had been excised upon re-integration into the tobacco genome.

*The dTam3 element has integrated into repetitive tobacco DNA, adjacent to a truncated T-DNA*

To establish the nature of the DNA flanking the re-integrated dTam3 element we used the flanking fragments as probes for tobacco DNA. The 0.8 kb *Eco* RV-*Xba* I fragment (Fig. 5A, probe A) containing dTam3 and flanking sequences was used as a probe to hybridize with the *Eco* RV-

digested DNA of plant 4202 and *Nicotiana tabacum* DNA. The hybridization pattern revealed that the flanking plant DNA sequences are repetitive sequences of the tobacco genome (Fig. 6). Surprisingly, the 0.8 kb *Pvu* II-*Eco* RV fragment flanking the other end of the dTam3 (Fig. 5A, probe B) did not yield the same hybridization pattern. Some of the hybridizing bands were also present in the NPTII hybridization pattern of plant 4202, but not in the parental plant DNA (data not shown). This suggested that the flanking sequence at this end also contained part of a newly integrated T-DNA fragment. Therefore we sequenced the DNA fragments flanking dTam3. The sequence data confirmed that the right end of a truncated T-DNA copy, containing the nos-NPTII fragment, flanked the dTam3 element (Fig. 5B). The *Eco*RV site at the right side of the pRIT65 fragment is not derived from T-DNA, but is derived from a small fragment of plant DNA. Apparently, the dTam3 element has integrated next to an incomplete T-DNA fragment. The dTam3 element has excised from the HPT gene of one T-DNA and re-integrated not further then two map units away, directly adjacent to a truncated T-DNA copy. The sequence of the junction between the dTam3 element and

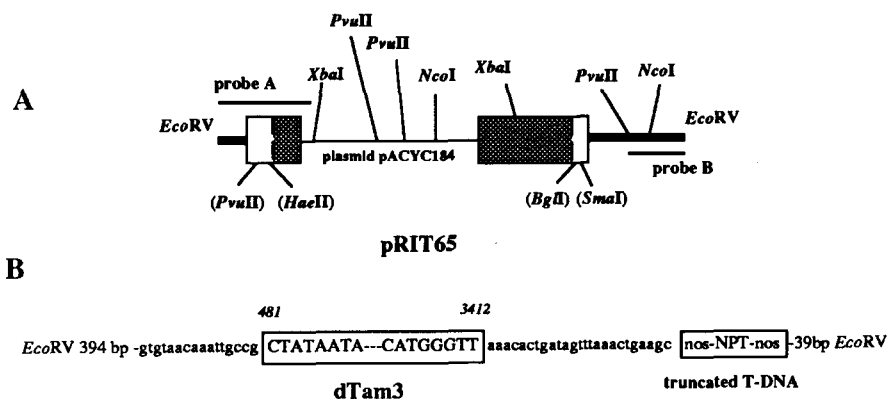


Fig. 5. Restriction endonuclease map of plasmid pRIT65, containing the re-integrated dTam3 element and its flanking sequence. A. Linear presentation of the re-integrated dTam3 element when digested with *Eco* RV. The 7 kb fragment contains 3.5 kb pACYC184 DNA with two Tam3 fragments flanking it. Both ends of the dTam3 element have been degraded (open boxes). The restriction endonuclease sites that have disappeared from the Tam3 fragment are indicated below the map in parenthesis. The DNA flanking dTam3 is represented by black bars. Fragments A and B used as probes in DNA gel blot analyses of tobacco DNA are indicated. B. Sequence of tobacco DNA (small caps) flanking the dTam3 fragment and a truncated T-DNA. Numbers above the dTam3 fragment refer to the published Tam3 sequence [15].

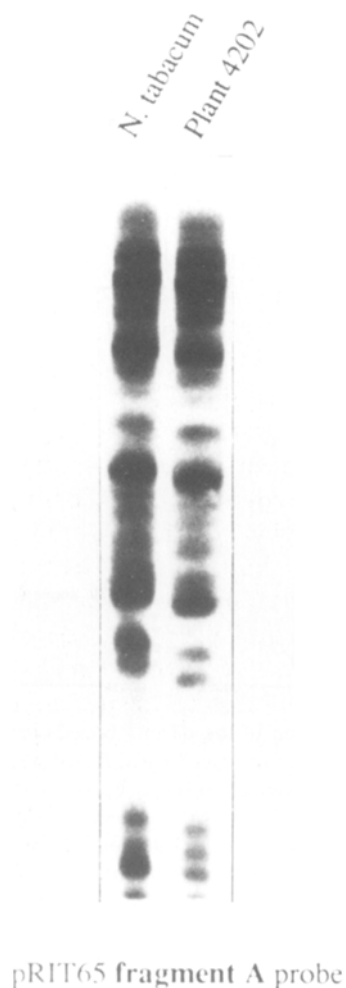


Fig. 6. The re-integrated dTam3 element is flanked by repetitive tobacco DNA sequences. Tobacco DNA from an untransformed *Nicotiana tabacum* plant and plant 4202 was digested with *Eco* RV. The DNA gel blot was probed with fragment A from plasmid pRIT65.

the tobacco DNA revealed that the dTam3 element had sustained a deletion of 207 bp at the right end. Comparison of the Tam3 sequence [15] and the sequence at the junction of the left flanking fragment and the dTam3 element demonstrated that 481 bp at the left end of the transposable element had been deleted. No target site duplication flanking the re-integrated dTam3 fragment was present (Fig. 5B). As target site duplications are a special feature of plant transposable element integration [3, 6], it can be envisaged that the re-integration of this dTam3 element

has been the result of a different integration process in tobacco.

## Discussion

The construction of an artificial Tam3 two-element system appears to be possible in a heterologous genetic background. An immobilized transposase donor Tam3 element and an internally deleted non-autonomous Tam3 element are both incapable of excision, as has been determined with a phenotypic assay [10]. When a plant containing either of the constructs is transformed with the second component of the *trans*-activation system hygromycin, resistant plants can be obtained at low frequencies (5% of the transformed leaf-discs yields a resistant shoot). Still, these results are significant, as control transformation experiments with plasmids pTT 21806, 21808 and 21840 on the same number of leaf discs from wild-type *N. tabacum* SR-1 plants did not yield any resistant shoots. The combination of the Tam3 derivatives can induce excision and restore the expression of the CaMV-HPT gene, although autonomous excision of the 'wings-clipped' element at a very low frequency could be detected by molecular assays. We can rule out artefacts resulting from T-DNA plasmid rearrangements as no PCR products could be detected in *Agrobacterium* DNA.

The excision of the dTam3 element from the HPT gene is confirmed by DNA gel blot analysis. The analysed plants have empty donor site fragments of different sizes. This is not unusual as Tam3 is known for the rearrangements it introduces in the empty donor site of the *pallida* and *nivea* locus of *Antirrhinum* [3]. However sequence analysis reveals that the variations in fragment size are not the result of rearrangement of the HPT gene, but are due to the presence of variable lengths of Tam3 DNA. Apparently excision of the dTam3 element from pTT21840 did not occur by a cut outside the transposable element but by internal excision. A more or less constant part of the right end of Tam3 (25–30 bp) remains in the empty donor site while a variable part of the left





On the other hand, internal excision is a well-known phenomenon of a transposable element structurally related to Ac and Tam3: the P-element from *Drosophila* [24, 30]. A recent report presents evidence for the suggestion that internal excision of a non-autonomous P-element only occurs when no wild-type locus is present (in the case of strains homozygous for a P-element insertion [5]). Precise restoration of the wild-type sequence is achieved by a homology-dependent gene conversion mechanism. The dTam3 excision products we obtained have similar features as internal excision products of P-elements. Internal P-element excision occurs when no wild-type homologue of the gene it is inserted into is present, it requires the presence of transposase, the sequence breakpoints are variable (15–20 bp inside the element) and are present at (or have generated) short direct duplications [5]. The dTam3 elements (inserted into the HPT gene) have no homologue (wild-type CaMV35S-HPT gene) in the tobacco genome and the excision products only occur in a two-element system with a transposase Tam3 $\Delta$ TIR element present. Furthermore, a CGG(C) motif is present as a direct duplication on either end of the breakpoint in four cases. The mechanism that explains the P-element internal excision is a double-stranded gap repair mechanism, that uses the homologous P-element as a template. The internal excision product is the result of an interrupted repair process. Only the ends of the homologous P-element have functioned as templates for repair, instead of restoring the complete P-element. The homologue-dependent gap-repair mechanism can also function on a homologous gene that is present elsewhere in the genome of *Drosophila*. The frequency of reversion is significantly lower in this case [5]. When the dTam3 elements are introduced into the transgenic tobacco plants no homologous dTam3 construct is present, but in all plants multiple copies of the T-DNA carrying the dTam3 (or Tam3 $\Delta$ TIR) element have integrated (results not shown). A possible double-stranded gap-repair mechanism, resulting in internal Tam3 excision products could therefore use the other T-DNA copies as templates. However, a contra-

diction with the P-element system comes from the analyses of re-integration. In the P-element system there still are new insertions in the genome while an internal excision is registered in the empty donor site [5, 30]. Of the six plants we analysed only one plant had a re-integrated non-intact dTam3 element. Therefore we cannot conclude that the dTam3 excision products result from a similar mechanism as the P-element internal excision. However, from the preceding discussion it might be concluded that the Tam3 excision is aberrant either due to an altered balance in the 'transposase' production by Tam3  $\Delta$ TIR or by the interaction with tobacco host factors during the transposition event.

The result of an altered excision specificity of dTam3 could be that the excised elements fail to re-integrate. This is apparently the case for most transformants, the only case of dTam3 re-integration being aberrant. The ends of the transposable element have been degraded and no target site duplication was found. Although it could be reasoned that this element is part of a truncated T-DNA this is very unlikely. Both the left end and the right end of the T-DNA would have to be lost during the integration process. It is more likely that this excised dTam3 fragment has benefitted from the integration process of a truncated right border T-DNA fragment (these two fragments are only separated by 24 bp of DNA), suggesting that this event occurred independent of transposon factors. This would fit in with the fact that these excision phenomena are observed upon or directly after integration of the T-DNA into the tobacco genome, as none of the plants examined is chimaeric for a transposition event and a non-transposed dTam3 element.

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